Chapter 1

Introduction

Part I
Epidermolysis bullosa simplex

Clinical features of epidermolysis bullosa simplex

Epidermolysis bullosa (EB) is a group of autosomally inherited skin diseases with an average prevalence of 1 in 17,000 individuals (Horn et al., 1997). The main clinical feature is fragility of the skin and mucous membranes after mild mechanical trauma, resulting in blister formation. Over twenty different subtypes of EB have been identified, each with their own characteristic symptoms. EB can vary from a relatively mild condition to a severe, even fatal, disorder.

According to the level of blister formation, three main groups of EB are distinguished: EB simplex (EBS), junctional EB (JEB) and dystrophic EB (DEB) (**Fig**. **1**). EBS is characterised by blister formation within the epidermis, whereas in JEB blister formation is seen at the level of the lamina lucida within the basement membrane zone and in DEB the split occurs at the level below the lamina densa in the papillary dermis.

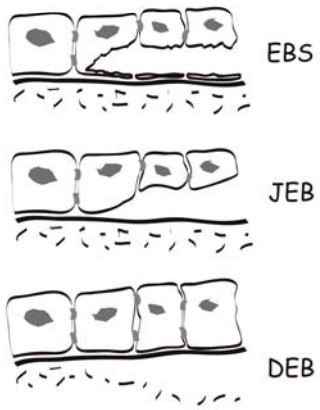

Figure 1 *Level of blister formation in different subtypes of epidermolysis bullosa. In epidermolysis bullosa simplex (EBS) blistering occurs at the level of the epidermis. In junctional epidermolysis bullosa (JEB) blister formation is seen at the level of the lamina lucida. Blister formation in dystrophic epidermolysis bullosa (DEB) occurs within the dermis.*

In EBS, three major subtypes can be discriminated according to clinical criteria (Fine et al., 1991) (**Fig. 2**). The most severe form, EBS herpetiformis, Dowling-Meara type (EBS-DM), is characterised by generalised skin blistering in a circinate pattern (originally annotated by the misnomer herpetiform) (**Fig. 2A**). The blistering in EBS-DM often causes keratoderma (thickening of the skin) on the palms of the hands and soles of the feet. At the electronmicroscopy level, clumps of keratin filaments can be identified in the basal keratinocytes (Anton & Schnyder, 1982; Niemi et al., 1983; McGrath et al., 1992). Milder forms are EBS-Koebner (K) and EBS-Weber-Cockayne (WC). In EBS-K, blistering is widespread over the body's surface but without the circinate pattern as in EBS-DM, whereas in EBS-WC blisters develop mainly on hands and feet (Haneke & Anton, 1982). In addition, a few other rare subtypes of EBS exist, including EBS with muscular dystrophy (EBS-MD) and EBS with mottled pigmentation (EBS-MP).

A. EBS-DM **B.** EBS-WC **C.** EBS-K

Figure 2 *Blister formation in the major subtypes of epidermolysis bullosa simplex.*

Molecular aspects of epidermolysis bullosa simplex and mutation detection

In the majority of families, EBS is inherited as an autosomal dominant disorder. In rare cases, however, the disease is transmitted in an autosomal recessive mode [recessive epidermolysis bullosa simplex, REBS] (Chan et al., 1994; Rugg et al., 1994; Jonkman et al., 1996).

The major subtypes of EBS (DM, K, WC) and also MP are caused by mutations, mostly nucleotide substitutions, in either of the keratin genes, *KRT5* (12q13) or *KRT14* (17q12-q21). EBS-MD, however, appears to be a distinct autosomal recessive disorder due to mutations in both alleles of the plectin (*PLEC1*) gene, leading to plectin deficiency (Smith et al., 1996).

The *KRT5* and *KRT14* genes encode the epidermal keratins K5 and K14, respectively. K5 belongs to the type II keratins, or basic keratins, and has a molecular weight of 58 kDa; K14 belongs to the type I keratins, or acidic keratins, and has a molecular weight of 50 kDa (Nelson & Sun, 1983; Conway & Parry, 1988). The keratins K5 and K14 are mainly expressed in basal keratinocytes, the basal cell layer of the epidermis (**Fig. 3**). Together these proteins form heterodimers. These dimers readily interact with each other in a staggered, antiparallel fashion to form stable tetramers, which assemble into the keratin filament network of the basal cells in the epidermis (**Fig. 4**) (Hatzfeld & Weber, 1990). Disturbances of the keratin filament network, caused by alterations in one of the keratin molecules, will lead to skin fragility and subsequently, after entering of fluid from the blood vessels to the intraepidermal space, blistering of the skin.

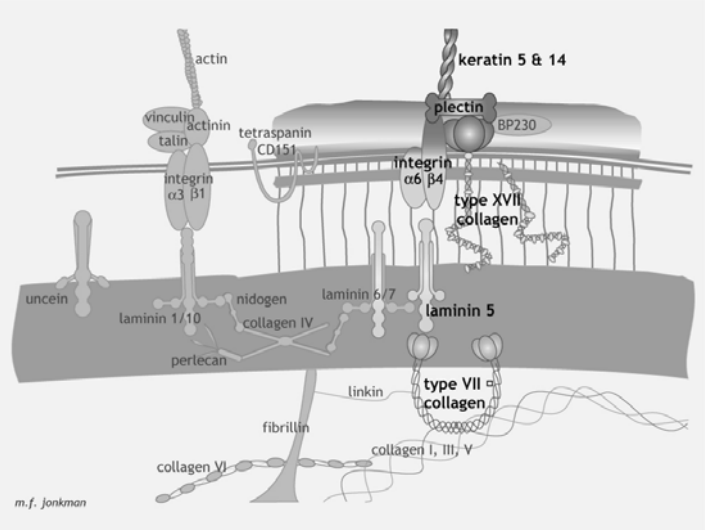

Figure 3 *Proteins of the basal membrane zone, including keratin 5 and keratin 14 of the epidermis, collagen XVII of the junction between the epidermis and the dermis and collagen VII in the dermis.*

Both keratins, K5 and K14, are composed of a central α-helical rod domain, flanked by nonhelical head and tail domains. The helical-rod is subdivided into helices 1A, 1B, 2A and 2B connected by three short nonhelical linker segments, linkers L1, L1-2 and L2, respectively (**Fig. 4**) (Conway & Parry, 1988). Most mutations reported to date appear to cluster in the *KRT5* exons 1, 5 and 7 and in the *KRT14* exons 1, 4 and 6 coding for the rod ends of 1A and 2B and the non-helical linker region L1-2 in both gene products (**Fig. 5**).

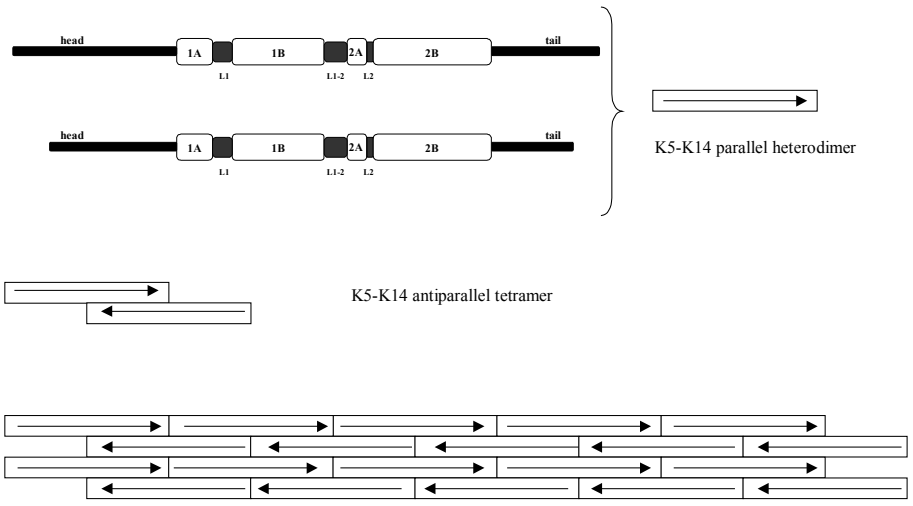

Figure 4 *Schematic picture of keratin filament formation. The filament structure is composed of dimeric molecules each containing a K5 and a K14 chain. These heterodimers readily interact with each other in a staggered antiparallel fashion to form stable tetramers, which assemble into the keratin filament network.*

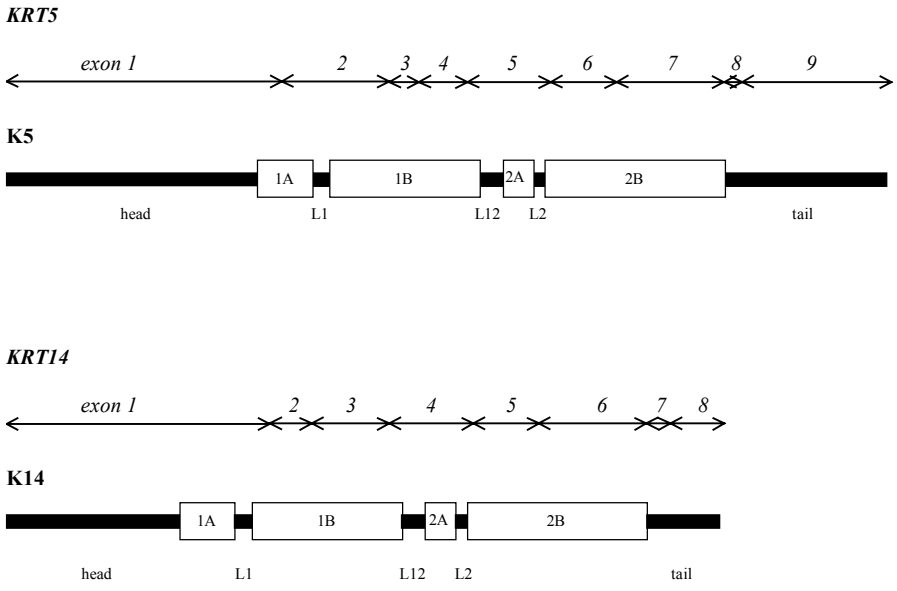

Figure 5 *Schematic picture of the relationship between the coding regions of the genes KRT5 and KRT14 with the domains of the proteins K5 and K14, respectively.*

For *KRT5*, a genomic mutation detection system has been described by Stephens et al. (1997). This system makes use of intronic primers for specific amplification of the nine *KRT5* exons that are subsequently directly sequenced. For *KRT14*, however, no such mutation detection system had been described since genomic mutation detection analysis is complicated by the presence of a highly homologous keratin 14 pseudogene. The exon and the intron sequences of the functional *KRT14* gene and the keratin 14 pseudogene are 95% and 93% identical, respectively. However, compared with the functional gene, the keratin 14 pseudogene shows some frameshift mutations, alterations in intron/exon boundaries and disruption of the polyadenylation signal (Savtchenko et al., 1988). Due to these mutations, the keratin 14 pseudogene is not transcribed or translated. Consequently, *KRT14* mutation analysis in EBS has mostly been carried out using cDNA synthesised from *KRT14* transcripts in mRNA isolated from skin biopsies.

Part II
Targeted gene correction

Gene therapy strategies are currently being developed for a wide spectrum of hereditary disorders, including epidermolysis bullosa. For autosomal recessive disorders, most approaches have been based on gene addition, i.e. episomal or genomic integration of a functional copy of the responsible gene. In the last few years, however, new strategies have been introduced attempting to correct or neutralise specific molecular defects at both the DNA and RNA level by interaction with specific oligonucleotides.

Gene therapy at the DNA level

Oligonucleotides with homology to a certain gene can interact with DNA to prevent transcription of that gene or to alter it by recombination or mismatch repair. Examples of such oligonucleotides include RNA/DNA-oligonucleotides [RDO], oligodeoxynucleotides [ODN] and triplex forming oligonucleotides [TFO] (**Table I**).

- RDO

RDOs as used to date are 68-mer single-stranded oligonucleotides capable of folding back on themselves to form a duplex structure. The molecule is composed of DNA residues with two intervening blocks of ten 2'-O-methylated RNA residues flanking a stretch of five DNA residues containing the desired base-change for correction of the defective gene. It is thought that the underlying mechanisms of RDO-mediated nucleotide conversion are recombination and mismatch repair. A number of publications describe successful correction with RDOs of specific point mutations in episomal and genomic DNA in mammalian cells (Cole et al., 1996; Yoon et al., 1996; Kren et al., 1997; Xiang et al., 1997; Alexeev & Yoon, 1998; Bandyopadhyay et al., 1999; Igoucheva et al., 1999), in plants cells (Zhu et al., 1999; Beetham et al., 1999) and in animals (Kren et al., 1998; Bandyopadhyay et al., 1999; Kren et al., 1999; Alexeev et al., 2000; Bartlett et al., 2000; Rando et al., 2000). Repair efficiencies in these studies varied between <0.01% and 48%, with the

majority of studies showing repair in approximately 10% to 20% of cases. Targeted gene repair using RDO has also been achieved in nuclear or cell-free extracts, although with a much lower repair efficiency of about 0.1% (Cole et al., 1999; Igoucheva et al., 1999).

- ODN

ODNs are small single-stranded or double-stranded DNA fragments complementary to the target DNA except for one or a few nucleotides in the middle, designed for the correction of the mutation under study. ODNs bind to a DNA strand, resulting in an alteration of the DNA sequence by mismatch repair or recombination. This approach has been shown to be effective in episomal repair in mammalian cell cultures (Colosimo et al., 2001; Igoucheva et al., 2001) and nuclear extracts (Igoucheva et al., 2001) as well as in the chromosomal repair in mammalian cell cultures (Kunzelmann et al., 1996; Goncz et al., 1998; Igoucheva et al., 2001; Kapsa et al., 2001) and animals (Goncz et al., 2001; Kapsa et al., 2001). Using ODN, targeted correction efficiencies of $5 \cdot 10^{-4}$% up to 20% were obtained. On average the repair efficiencies varied between 0.1% and 1%.

- TFO

A TFO binds to a double-stranded DNA target in a sequence-specific manner in the major groove of the DNA helix to form a triple helix structure. This structure prevents the transcription of a gene under study by inhibiting transcription factor-binding or by interfering with formation of the initiation complex. A major limitation of this triplex technology is the sequence restriction of the target sequence that must consist of a sufficiently long stretch of purines for triplex formation. A variation of this strategy is the use of a tethered donor-TFO (TD-TFO) that consists of two different domains, a triple helix for binding to the sequence nearby the targeted base and a repair domain which is identical in sequence to the target gene, except for the desired sequence change. It has been shown that a TD-TFO could mediate episomal correction in mammalian cells with an efficiency of 1% (Chan et al., 1999), and both in cell free extracts (Datta et al., 2001) and in animals (Vasquez et al., 2000) with an efficiency of 0.05%.

Table I *Reported targeted gene correction*

					repair efficiency (%)	reference
RDO	mammalian cells	CHO-k1	episomal	alkaline phosphatase	30	Yoon et al., 1996
		CHO-k1	episomal	*LacZ*	0.1 - 1	Igoucheva et al., 1999
		CD34+ cells	chromosomal	β-globin	5 - 11	Xiang et al., 1997
		lymphoblastoid cells	chromosomal	β-globin	20	Cole et al., 1996
		HuH-7	chromosomal	alkaline phosphatase	11.9	Kren et al., 1997
		rat hepatocytes	chromosomal	factor IX	19 - 24	Bandyopadhyay et al., 1999
		melanocytes	chromosomal	tyrosinase	0.01 - 15	Alexeev & Yoon, 1998
	animals	mdx mouse	chromosomal	dystrophin	10 - 20	Rando et al., 2000
		Gunn rat	chromosomal	UDP-glucuronosyltransferase	20	Kren et al., 1999
		rat	chromosomal	factor IX	18	Kren et al., 1998
		dog	chromosomal	dystrophin	?	Bartlett et al., 2000
		rat	chromosomal	factor IX	48	Bandyopadhyay et al., 1999
		mouse	chromosomal	tyrosinase	?	Alexeev et al., 2000
	plant cells	maize cells	episomal	*AHAS*	0.0001	Zhu et al., 1999
		tobacco cells	chromosomal	*ALS*	?	Beetham et al., 1999
	nuclear extracts	CHO-k1	episomal	*LacZ*	0.1	Igoucheva et al., 1999
		HuH-7	episomal	tetracycline	0.1	Cole et al., 1999
ODN	mammalian cells	CHO-k1	episomal	*LacZ*	1	Igoucheva et al., 2001
		bronchial epithelial cells	episomal	zeocin	4	Colosimo et al., 2001
		CHO-k1	chromosomal	*LacZ*	0.1	Igoucheva et al., 2001
		airway epithelial cells	chromosomal	*CFTR*	1	Goncz et al., 1998
		airway epithelial cells	chromosomal	*CFTR*	1	Kunzelmann et al., 1996
		myoblast cells	chromosomal	dystrophin	15 - 20	Kapsa et al., 2001
	animals	mdx mouse	chromosomal	dystrophin	0.0005 – 0.1	Kapsa et al., 2001
		mouse	chromosomal	*CFTR*	?	Goncz et al., 2001
	nuclear extracts	DT40	episomal	*LacZ*	0.02 – 0.05	Igoucheva et al., 2001
TFO	mammalian cells	COS-7	episomal	*supFG1*	1	Chan et al., 1999
	animals	mouse	chromosomal	*supFG1*	0.05	Vasquez et al., 2000
	nuclear extracts	HeLa	episomal	*supFG1*	0.05	Datta et al., 2001

Gene therapy at the RNA level

There is also a group of molecules that interfere with gene function at the RNA level. These molecules prevent translation or interfere with RNA molecules. Examples are antisense molecules (Galderisi et al., 1999), PNA (Good & Nielsen, 1997), and ribozymes (Bramlage et al., 1998). Antisense molecules block or decrease the expression of specific target genes by binding to the mRNA molecules. The same effect can be obtained with PNA molecules. PNA (peptide nucleic acid) is a DNA mimic in which the phosphodiester backbone has been replaced by a polyamide (peptide) backbone. Binding of these molecules to mRNA either prevents the ribosomal complex from translating the sequence or causes RnaseH to bind to mRNA resulting in the degradation of RNA. Ribozyme molecules are catalytic RNAs that take care for the sequence-specific cleavage of mRNA.

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